PEPTIDE ANALYSIS USING TANDEM MASS SPECTROMETRY

by

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FINAL REPORT

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INTRODUCTION

The objective of the project was to determine the complete amino acid sequence of the large polypeptide Ubiquitin by use of fast atom bombardment (FAB) ionization and tandem mass spectrometry. The peptide containing 76 amino acid residues was available at Warwick University as 12 tryptic digests which had previously been separated by off-line reverse phase HPLC. Most of the peptides resulting from tryptic digest contained four to ten amino acid residues and were of molecular weights below 2,000 daltons.

Tandem mass spectrometry can be used to sequence peptides since the ions produced by FAB ionization can be selected individually by the first mass spectrometer and then induced to decompose after collision with a collision gas in a collision cell. These fragment ions are formed as the result of cleavages along the backbone of the peptide chain or due to the loss of specific amino acid side chains. The second mass spectrometer can then detect these daughter ions of a chosen parent ion. In this way tandem mass spectrometry with the use of collision induced decomposition can provide detailed structural information on the sequence of amino acids of the various tryptic fragments derived from Ubiquitin. The use of a floated collision cell maximizes the transmission of low mass daughter ions and leads to increased detection efficiency because of the extra kinetic energy imparted to the daughter ions after their formation in the collision cell. The use of the floated collision cell in the new four sector instrument was the primary objective in the peptide sequencing project.

Due to the fact that the four sector instrument did not arrive at Warwick University until February 1989 and was not operational until March 1989, preliminary work on peptide sequencing was done using the two sector Kratos MS50 mass spectrometer. This instrument which was outfitted with a collision cell which was not floated above ground was used to analyze most of the peptides which were then subsequently studied using the four sector instrument.

The introduction system described in this report was in all cases by a direct insertion probe with FAB ionization. Use of on-line microbore HPLC as a method of introducing peptide mixtures into the source of the mass spectrometer was demonstrated on the MS50 instrument during a one-day demonstration of the Gilson Model 305 precision HPLC pump by the Anachem company. The four sector instrument is not presently designed to accept the continuous flow FAB probe and an HPLC pump was not available at Warwick University for the MS50 aside from that loaned during a one day demonstration during February 1989.

The HPLC inlet system which we designed in house consisted of a capillary column splitter after the LC column which reduced the flow rate from 80 μL/min set by the HPLC pump to 8 μL/min into the source of the mass spectrometer. The design was successful but the data system of the MS50 did not allow for scanning of peptide ions eluting from the mass spectrometer.

The instrumentation described in this report consisted of a Kratos Concept II HH hybrid mass spectrometer (See Figure 1).

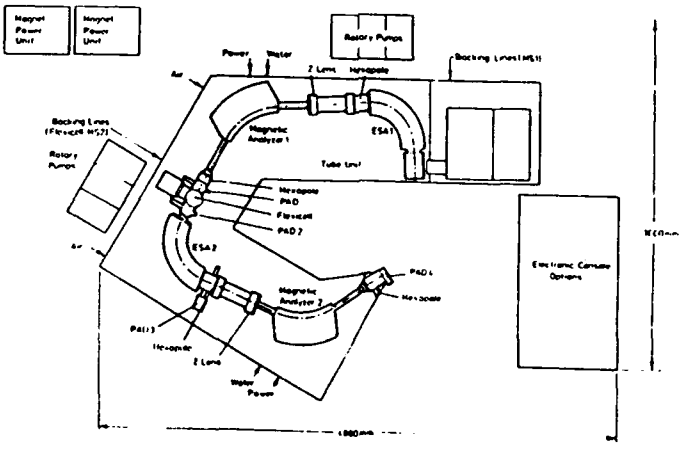


Figure 1. Overall layout of the Concept II HH system.

It is designed so that ions formed in the source are accelerated into an electrostatic analyzer (ESA1) which consists of two cylindrical plates maintained at voltages of + 500 V and - 500V so that only ions possessing 8 keV of translational energy pass through the electrostatic analyzer. The first mass analysis is set at a particular magnetic field strength so as to transmit ions of a chosen m/z. At a specific magnetic field strength of MS1, ions of only one mass are allowed to pass into the collision cell. There are continuously tunable, computer driven slits at the source exit, the end of ESA1 and the end of MS1 so that a high resolution is achieved. This allows only one of the isotope peaks of the peptide ion isotope cluster to pass into the collision cell. This simplifies the interpretation of subsequent fragmentations.

The collision cell at the end of the first mass spectrometer is an integral part of the Flexicell. (See Figure 2)

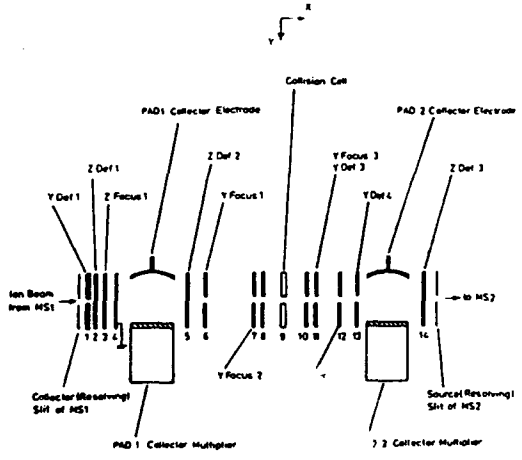
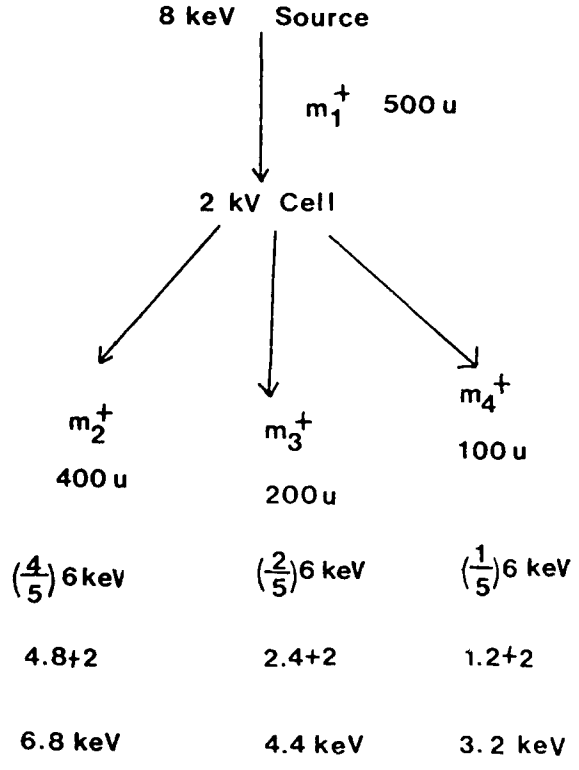


Figure 2. Arrangement of slit plates and post acceleration detectors in the Flexicell.

The flexicell is analogous to a second source for the second mass spectrometer and is used as such when calibrating it. For peptide sequencing work the importance of the flexicell is that a highly resolved beam of mass analyzed ions passes into the collision cell and on collision with rare

gas atoms some of the translational collisional energy is transferred into vibrational energy within the bonds of the peptide ions.

If the energy of this excited vibrational state exceeds the activation energy for fragmentation, daughter ions are produced with a range of masses which result in a range of kinetic energies for the new ions produced in the collision cell.



In order better to collect fragment ions formed from the parent ion the collision cell is floated at a potential 2kV above ground thereby reducing the collision energy to 6 keV. (See Figure 3)

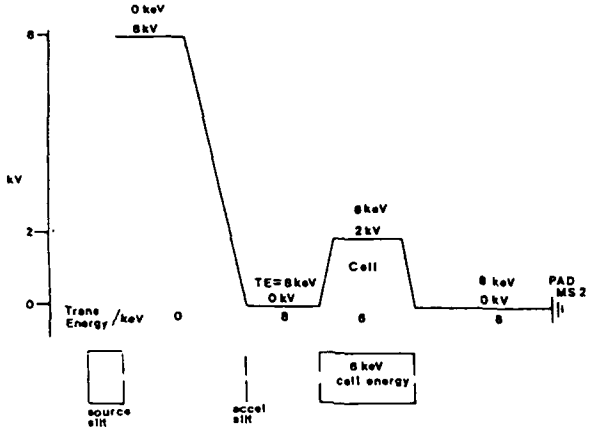


Figure 3. *Potential diagram showing* the variation in electrical potential throughout the four sector mass spectrometer with a floated collision cell.

Since the final potential at the detector of MS2 is at ground, the ions which are formed within the floated collision cell gain an extra 2 keV of translational energy as they fall from a potential of 2 kV back to 0 kV at the collision cell exit slit. Daughter ions of low mass will have lower translational energies than their parent ion so this extra translational energy gained by floating the collision cell improves the collection efficiency at the final post acceleration detector at MS2. Also, low mass ions are influenced by the fringing fields (like the lines of force around a bar magnet) of the subsequent magnet MS2. The increased translational energy reduces the deflecting influence of these fringing fields and enhances collection efficiency. The ion lens system out of the flexicell also improves ion focussing before ESA2.

In order to observe daughter ions formed from the collision induced dissociation of a specific parent ion chosen at the end of the first mass spectrometer, the second mass spectrometer must be scanned in a dynamic B/E scan.

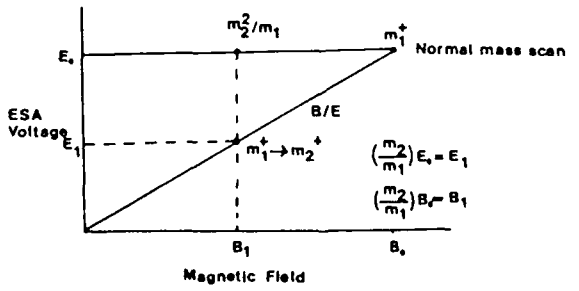
An electrostatic analyzer is an energy analyser. When daughter ions are formed upon collision the decrease in mass results in a loss of translational energy. Consequently the daughter ion will have a new energy translational and will only pass through the electrostatic analyzer if the electric sector voltage is reduced in proportion to the decrease in mass:

$$E_2 = \left(\frac{m_2^+}{m_1^+}\right) E_0$$

A magnetic analyzer is a momentum analyzer. When daughter ions are formed upon collision they will have a new momentum, lower than that of the parent ion due to the decrease in mass. Ions will only pass through the magnetic analyzer if the magnetic field strength is lowered in proportion to the mass decrease:

$$B_2 = \left(\frac{m_2^+}{m_1^+}\right) B_0$$

In a normal mass spectrum the voltage of the electrostatic analyzer is maintained at a constant potential and only those ions of a specific translational energy (8 keV in these experiments) can pass through to the magnetic analyzer. The magnetic analyzer is then scanned from high magnetic field strength to low magnetic field strength and ions of various masses are brought to focus at the collector.



A daughter ion derived from decomposition in a field free region will have an apparent mass $m^* = m_2^2/m_1$. In order to detect all daughter ions formed from a particular parent ion the electrostatic and magnetic field strengths must be lowered in tandem after having focussed the parent ion initially at a particular electrostatic field voltage and magnetic field voltage.

The linked scan law predicts that once the parent ion has been focussed at the collector at a particular value E_0 and B_0, a linear decrease in B and E at a constant ratio will bring into focus all daughter ions originating from the initial parent ion (1). The disadvantage in the use of a floated collision cell is that although it results in improved daughter ion transmission and increased sensitivity, it results in a more complex form of the linked scan law so that the B/E scan is no longer linear (2). The normal scan law is derived for transmission of a precursor ions into the collision cell at full kinetic energy and momentum. If the scan function has been calibrated in terms of mass for ions of full kinetic energy, this function will not apply when the fragment ion is formed in a cell above earth potential. The scan law for the B/E scan becomes more complex when the collision cell is floated at 2 kV and deviates even more from linearity when the collision cell is floated at 7.5 kV. The computer will calculate this new scan law with each new value of collision cell voltage selected and an example of this is shown in Figure 4 and Figure 5.

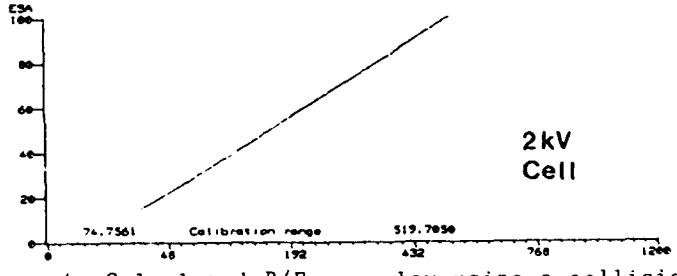


Figure 4. Calculated B/E scan law using a collision cell floated at 2 kV.

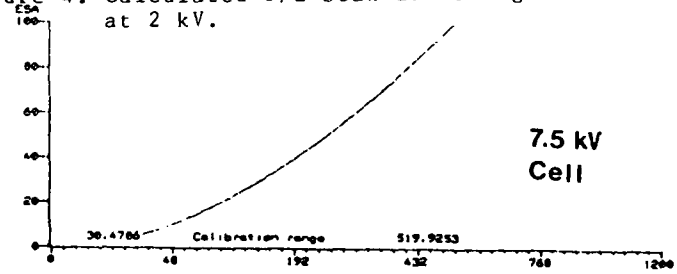


Figure 5. Calculated B/E scan law using a collision cell floated at 7.5 kV.

The collision induced decomposition spectra described in this report were obtained using the four sector mass spectrometer equipped with a floated collision cell described above and with the use of a two sector MS50 mass spectrometer described below.

The MS50 instrument is shown in Figure 6.

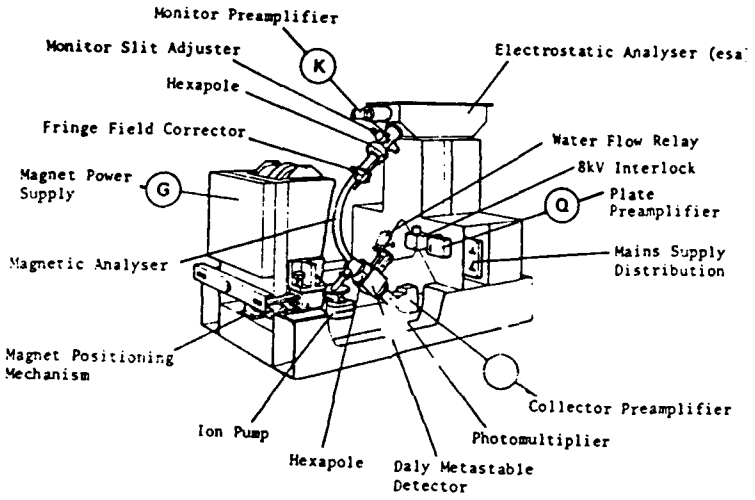


Figure 6. Tube unit of the Kratos MS50 mass spectrometer.

It consisted of a FAB source and FAB gun which was used with accelerated Xenon gas as the bombarding atom beam, a collision cell located just behind the source slit, an electrostatic analyzer which allowed the passage of ions having 8 keV translational energy and a magnetic analyzer which was a water cooled extended range magnet allowing for a mass range of 1750 at 8 kV accelerating voltage. Since the MS50 instrument had no Hall probe converter allowing for computer control of the magnet position, the experiments performed using linked scans had to be done in a very different way from the B/E scans obtained on the four sector instrument which operated under field control using a Hall probe. The collision gas inlet system on the MS50 consisted of a glass high vacuum manifold from which two different collision gases could be introduced in rapid succession. In this way the collision gas could be rapidly switched

without changing any of the source focus settings and a comparison of the efficiency of daughter ion production of two gases (argon or helium) could be made. Although helium has been widely used as the collision gas, primarily because scattering of the primary ion beam is minimized, the conversion of translational energy to internal energy is relatively low so that the extent of fragmentation is limited. Argon, being a heavier target has a larger intrinsic CID cross section and is thought to be more effective at producing daughter ions from high energy collisions. Part of the objective of the MS50 work was to obtain some quantitative data on this subject on which there is still a controversy in the literature (3).

EXPERIMENTAL PROCEDURE

The procedure for obtaining a collision induced dissociation spectrum of a peptide using the 4-sector tandem mass spectrometer involves a series of tuning steps prior to the actual experiment in which one optimizes transmission of the parent ion, decreases the parent ion beam by collisions in the cell and then tunes for maximum transmission of daughter ions through MS2. All of this requires several minutes. Since the ubiquitin peptides are available in limited quantities and last on the FAB probe tip for only 5 - 10 minutes (even when using an aerosol coolant pumped into the probe tip) the initial tuning is done with a commercial sample of Bradykinin (MH^+ = 1060.6). The instrument is first calibrated over ESA1 and MS1 using a mixture of CSI and RbI. The second mass spectrometer is calibrated by inserting the Phasor FAB source at the collision cell direct insertion lock. A CSI/RbI spectrum is obtained using MS2 only and this calibrates the second mass spectrometer.

Bradykinin (1 - 5 nmol) is then introduced into the FAB source of MS1 and the post acceleration detector at the end of MS1 is used to obtain maximum ion intensity by tuning on the source focus knobs. Then the data system is directed to monitor the detector at the end of ESA2 and the flexicell settings are optimized. Subsequently the detector at MS2 is selected and a narrow scan of the ESA2 voltage is obtained so as to transmit ions of slightly different momenta. One then tunes again on the flexicell settings and tunes for maximum signal intensity at the detector of MS2.

At this point a collision gas is added and while monitoring the intensity of the parent ion beam at MS2, the gas pressure is increased until 40% of

the original peak intensity remains. A fragment ion mass is then selected and the detector at MS2 is used while one optimizes for transmission of this daughter ions out of the flexicell by tuning on the flexicell settings. The flexicell is designed so that a collision cell potential of 2 kV is the optimum value for obtaining low mas fragments.

Finally, the Ubiquitin peptide sample is introduced into the source. The collision gas is turned off and the parent ion beam is detected at the end of MS1 and then MS2. A minimum amount of refocussing may be necessary. The collision gas is reintroduced and the signal decreased by 60%. One then directs the data system to perform a metastable calibration over MS2 to allow the passage of all daughter ions arising from the peptide parent. Once the data system has calculated the B/E linked scan law for MS2 using a collision cell floated at 2kV, data acquisition is initiated. In certain cases the sensitivity is increased by acquiring the B/E data as raw uncentroided peaks. In these cases several B/E scans can be summed and the scans subsequently mass assigned by use of a time to mass reference file.

The collisional activation experiment usingthe MS50 two sector instrument is somewhat simpler than the four sector experiment. When focussing on the parent ion of the Ubiquitin peptide it is necessary to tune the source controls of the MS50 for maximum sensitivity of the parent ion and add the collision gas to decrease the intensity of the beam by 60%. The collision gas inlet value is then closed. The peptide sample is then removed from the instrument, a calibrant (usually a mixture of LiI, CsI and RbI) introduced and a low resolution calibration is performed. The peptide sample is then reintroduced into the FAB source and an accurate determination of the mass of the parent ion to two decimal places is obtained after which a metastable calibration is performed. This calibration allows the computer to calculate exactly how to drop the magnetic field strength (B) and the electrostatic field strength (E) simultaneously to observe all fragment ions which are derived from the parent peptide ion. Since the MS50 operates under current control rather than the Hall probe monitored field control and does not have a digital to analogue converter to set the magnetic field strength value from the computer, it is not possible to tune on the daughter ion peaks once a calibration has been performed. Data were acquired as centroided peaks

which were mass assigned by a computer generated time/mass file.

RESULTS AND DISCUSSION

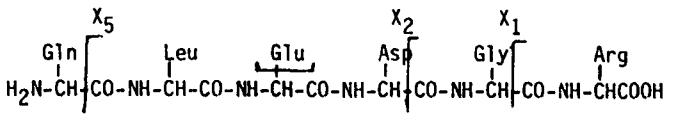
Ubiquitin tryptic fragment 4 elutes from a reverse phase HPLC column as the fourth peak in a complex mixture. It is proposed to have the amino acid sequence (Gln-Leu-Glu-Asp-Gly-Arg) and a theoretical molecular weight for the protonated molecular ion, MH^+ of 717.35.

Spectrum 1 is a normal mass scan of Ubiquitin fragment 4 obtained by fast atom bombardment ionization using the MS50 mass spectrometer. The measured mass of the largest peak of the parent ion isotope cluster was 717.32. The magnet scanning rate was 30 seconds/decade and the source accelerating voltage was 8kV. The prominent peak at m/z 700 is due to cyclisation of the N-terminal GLU to give pyroglutamic acid during storage, eliminating ammonia. It is almost absent in the spectrum of a freshly-prepared sample and is not seen in the B/E linked scan spectrum of the m/z 717 ion (see below).

Spectrum 2 is a B/E scan of the same peptide obtained immediately after Spectrum 1. The parent ion at mass 717.32 was decreased in intensity by 60% using helium as a collision gas. The pressure of helium gas was 60 mbar measured at the collision cell inlet pressure gauge. Daughter ions were formed with equal intensity over a large mass range. The spectrum can be interpreted as originating from the loss of amino acid side chains from the parent ion with only some peptide backbone cleavages.

The manner in which a peptide ion can fragment has been outlined as a series of backbone cleavages which fall into six classes (4). The ions formed when the charge is retained at the N-terminal (amino) peptide are termed A, B and C ions while those formed where charge is retained at the C-terminal (carboxyl) end are called X, Y and Z ions.

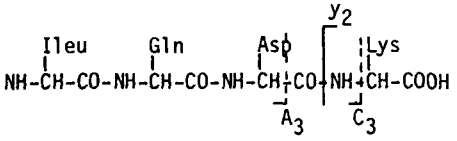
The cleavage of Ubiquitin fragment 4 indicated by the collision induced dissociation spectrum shown in Spectrum 2 can be described as follows:



The Ubiquitin fragment 4 obtained using helium as a collision gas on the 4-sector Concept mass spectrometer is shown in Spectrum 3. The data were obtained as normal centroided peaks and in a single scan of 30 seconds/decade. The collision cell voltage was 2kV. The source accelerating voltage was 8kV. The sample which was dissolved in a matrix of glycerol/acetic acid was bombarded with a beam of xenon atoms at 6kV. There are many more daughter ion peaks detected in this experiment than those detected using the MS50 equipment. The fragmentation occurs much more along the peptide backbone. While several of the predicted A, B, C and X, Y Z ions are observed there are several prominent ions due to loss of amino acid side chains.

Ubiquitin fragment 2a is a tryptic digest fragment which elutes as the second peak in the HPLC elution chromatogram. It corresponds to amino acids 30 - 33 in the intact Ubiquitin sequence and has a theoretical mass for the protonated molecular ion MH^+P of 503.28. The actual parent ion mass measured was 503.05 (spectrum not shown).

The amino acid sequence of this peptide is:

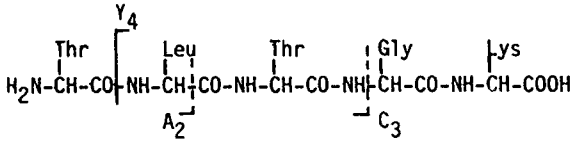


Spectrum 4 is the collision induced fragmentation of Ubiquitin fragment 2a observed using the MS50 mass spectrometer. The collision gas was argon which was added to decrease the parent ion beam intensity by 50%. There are several ions observed at MH-1, MH-2, etc. The daughter ions resulting from backbnond fragmentations are few in number and of relatively low intensity.

Spectrum 5 shows the collision induced fragmentation of Ubiquitin fragment 2a using the Concept 4 sector mass spectrometer at a collision cell voltage of 2kV with helium gas. Data were acquired as centroided peaks at 5 seconds/decade scan speed. The matrix used was glycerol/acetic acid. The daughter ions resulting from backbone cleavages are observed over a

large mass range provide information on backbone cleavages as well as loss of specific amino acid side chains.

Ubiquitin fragment 3 elutes as the third major peak in the tryptic digest HPLC chromatogram. It corresponds to amino acids 7-11 of the intact Ubiquitin peptide. It has a theoretical mass of 519.31 and an amino acid sequence:



Spectrum 6 is the collision induced dissociation of Ubiquitin fragment 3 using the Concept 4-sector mass spectrometer. The collision gas was helium which was used to decrease the parent ion beam intensity by 60%. The data was acquired as raw uncentroided peaks. The sum of five scans which was subsequently mass assigned placed the parent ion peak outside of the calibration range (slightly above 519.9u). The collision cell voltage was 2kV. The source accelerating voltage was 8kV. The sample matrix was acetic acid/glycerol. A beam of fast xenon atoms accelerated to 6kV was used to ionize the sample.

The large number and high intensity of daughter ions observed in Spectrum 6 provides confirmation of the proposed amino acid sequence of Ubiquitin fragment 3. A, C and Y ions occur with significant intensity across a very large mass range. It is particularly important that intense ions are seen in the low mass region at 89u and 188u. This is evidence of the enhanced sensitivity which can be achieved when using a floated collision cell. The extra translational energy given to the low mass daughters as they exit the flexicell allows them to be transmitted through the second mass spectrometer and detected by MS2 with greater efficiency.

The MS50-CID spectra obtained (Spectra 2,4) exhibit an intensity of low mass daughters at about one fifth that of the 4-sector spectra. The collision cell of the MS50 can be floated but this was not done in any of the experiments described in this report. The collision energy in the MS50 is higher than that in the four sector collision cell (8keV vs 6keV) but the expected increase in collision efficiency is offset by the less efficient collection efficiency of the MS50 instrument.

CONCLUSIONS

Since only three of the twelve tryptic fragments of Ubiquitin were analyzed by tandem mass spectrometry, the complete amino acid sequence cannot be determined from the experiments described in this report. If time had allowed it would have been possible to look at all of the twelve peptides using the four sector mass spectrometer at various collision cell energies. This would have given us much more information on the amino acid sequence of the individual tryptic fragments.

The problem remains that the order in which the fragments occur in the overall primary sequence cannot be determined by tandem mass spectrometry alone. Future experiments would require another enzyme digest of Ubiquitin with an enzyme such as chymotrypsin which cleaves the peptide at sites other than the tryptic cleavage sites. This would yield another set of fragments which could be sequenced by tandem mass spectrometry. One could then look for overlapping sequences in the two sets of digests to determine the overall primary sequence of the peptide.

The value of collision induced dissociation and tandem mass spectrometry in the sequencing of peptides resides in its extremely high sensitivity and the large amount of quantitative information which can be extracted from a very small sample size. It is a powerful analytical technique which along with high resolution chromatography and enzyme hydrolyses can yield the complete amino acid sequence of unknown peptides. It also provides a rapid method of checking amino acid sequences determined by more classical techniques.

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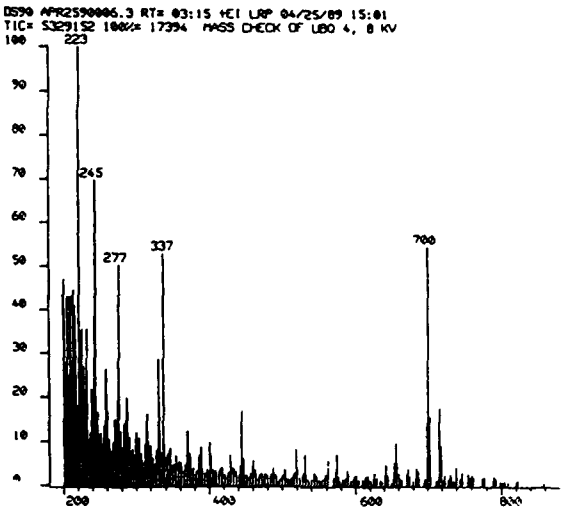
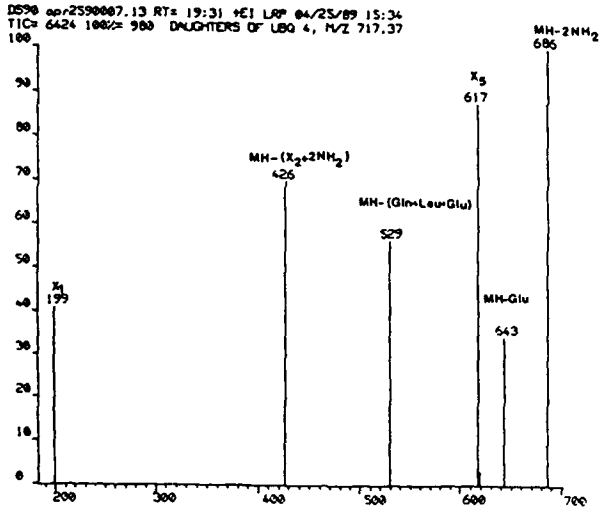
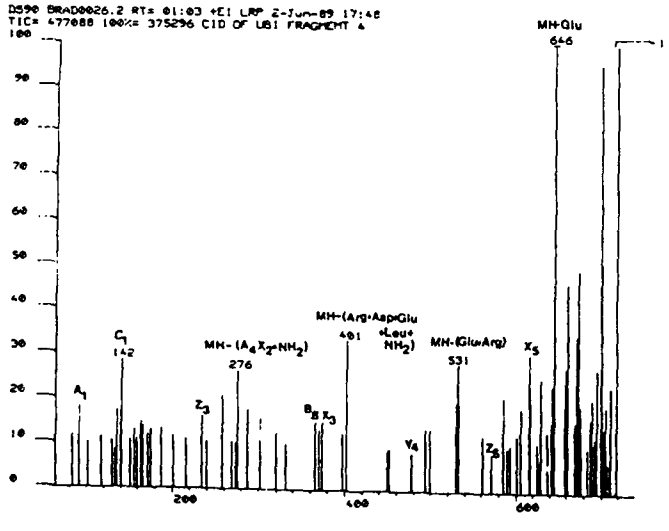


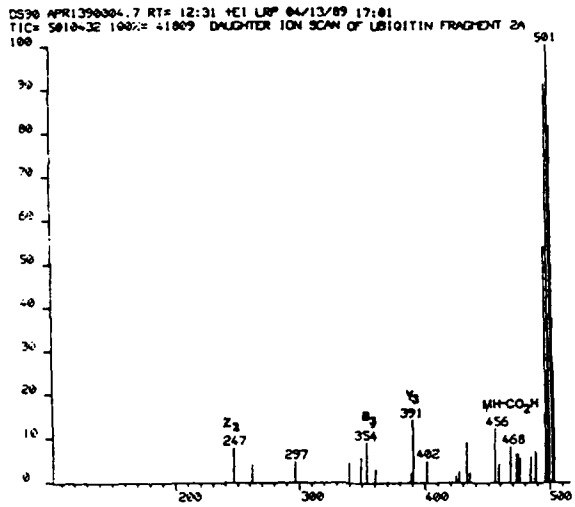
Spectrum 1. FAB mass spectrum of Ubiquitin fragment 4, MH^+ = 717.35 u (Gln-Leu-Glu-Asp-Gly-Arg).



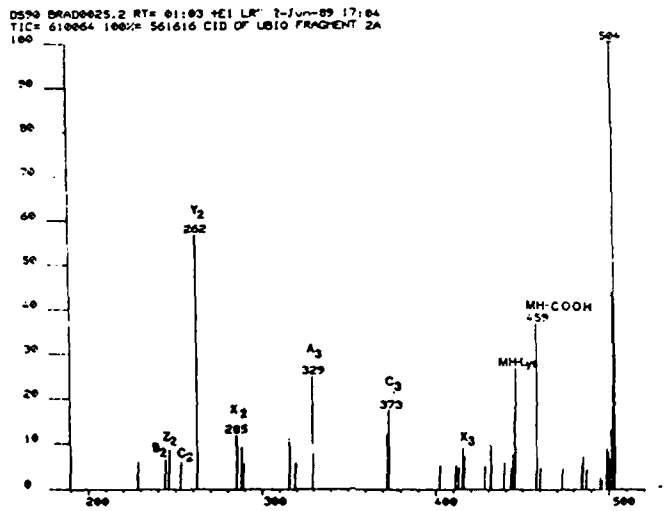
Spectrum 2. B/E linked scan of Ubiquitin fragment 4, MH^+ = 717.37 u . Spectrum obtained using MS50 mass spectrometer. Helium collision gas, 60% parent ion beam attenuation.



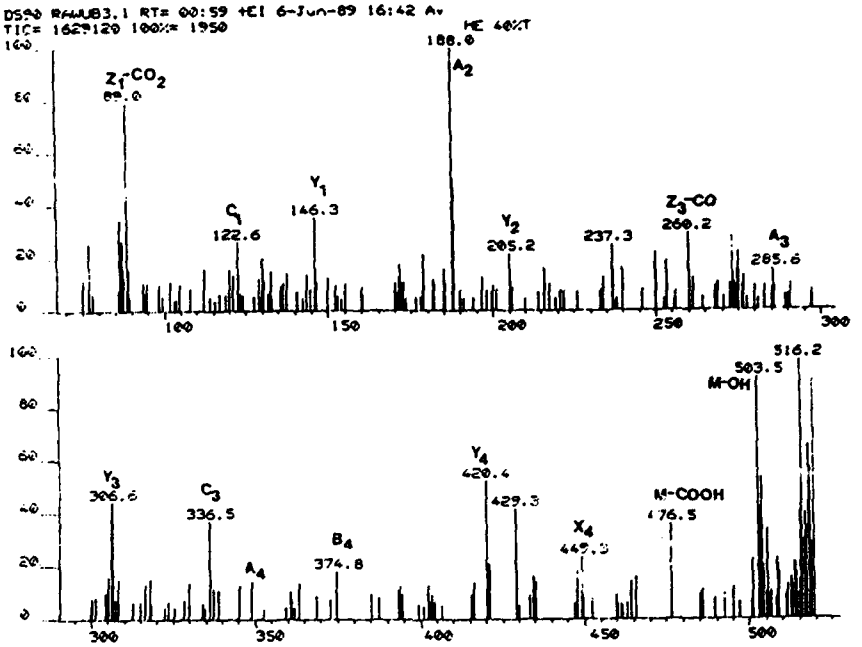
Spectrum 3. CID spectrum of Ubiquitin fragment 4 acquired using the Concept (EBEB) four sector mass spectrometer. Helium collision gas, 60% attenuation.



Spectrum 4. CID spectrum of Ubiquitin fragment 2a (Ileu-Gln-Asp-Lys), MH^+ = 503.28. Spectrum acquired using MS50 with argon collision gas, 50% beam attenuation.



Spectrum 5. CID of Ubiquitin fragment 2a acquired using the Concept EBEB four sector mass spectrometer. Single scan, 30 sec/decade. Helium collision gas, 60% attenuation.



Spectrum 6. Collision induced dissociation spectrum of Ubiquitin fragment 3 (Thr-Leu-Thr-Gly-Lys), MH^+ = 519.31. Five scans were acquired as uncentroided peaks, signal averaged and then mass assigned. Helium collision gas, 60% attenuation.